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Journal of Cellular Biochemistry 113:1132–1141 (2012)

Journal of Cellular Biochemistry

# Analysis of the Effects of HIV-1 Tat on the Survival and Differentiation of Vessel Wall-Derived Mesenchymal Stem Cells

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## ABSTRACT

HIV infection is an independent risk factor for atherosclerosis development and cardiovascular damage. As vessel wall mesenchymal stem cells (MSCs) are involved in the regulation of vessel structure homeostasis, we investigated the role of Tat, a key factor in HIV replication and pathogenesis, in MSC survival and differentiation. The survival of subconfluent MSCs was impaired when Tat was added at high concentrations (200–1,000 ng/ml), whereas lower Tat concentrations (1–100 ng/ml) did not promote apoptosis. Tat enhanced the differentiation of MSC toward adipogenesis by the transcription and activity upregulation of PPARγ. This Tat-related modulation of adipogenesis was tackled by treatment with antagonists of Tat-specific receptors such as SU5416 and RGD Fc. In contrast, Tat inhibited the differentiation of MSCs to endothelial cells by downregulating the expression of VEGF-induced endothelial markers such as Flt-1, KDR, and vWF. The treatment of MSCs with Tat-derived peptides corresponding to the cysteine-rich, basic, and RGD domains indicated that these Tat regions are involved in the inhibition of endothelial marker expression. The Tat-related impairment of MSC survival and differentiation might play an important role in vessel damage and formation of the atherosclerotic lesions observed in HIV-infected patients. J. Cell. Biochem. 113: 1132–1141, 2012. 

**KEY WORDS:** Tat; HIV-1; MESENCHYMAL STEM CELLS; DIFFERENTIATION; APOPTOSIS

HIV-1 Tat protein is a viral protein that plays a pivotal role in HIV replication [Dayton et al., 1986]. Tat binds the transactivation region (TAR) in the HIV long terminal repeat (LTR) affecting RNA polymerase activity and function through its interaction with specific cellular factors [Veschambre et al., 1995]. This multimeric protein complex augments the processivity of the RNA polymerase, providing a strong RNA elongation activity that dramatically increases HIV replication effectiveness and the number of HIV long mRNA transcripts [Laspia et al., 1989]. Beside these direct influences on HIV replication, Tat affects both uninfected and infected cells through paracrine and autocrine mechanisms due to the release of Tat in the extracellular medium by cells acutely infected with HIV-1 [Frankel and Pabo, 1988]. Extracellular Tat can bind several cell membrane receptors such as Flt-1, KDR, integrin, heparan sulfates, and chemokine receptors at different affinities [Albini et al., 1996; Mitola et al., 1997;

The authors declare that they have no competing interests.

Grant sponsor: Fondazione Cassa di Risparmio Bologna, Italy; Grant number: 2006.0035; Grant sponsor: Fondazione del Monte di Bologna e Ravenna, Italy; Grant number: 376 bis/2009; Grant sponsor: Italian Ministry of Health; Grant number: 40H5; Grant sponsor: University of Bologna, Funds for selected research topics of the University of Bologna and MURST 60%; Grant number: RFO09GIBEL.

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Received 1 November 2011; Accepted 2 November 2011 • DOI 10.1002/jcb.23446 • © 2011 Wiley Periodicals, Inc.
Published online 17 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

Gibellini et al., 2005a] activating different signal transduction pathways and transcription factors that, in turn, modulate the expression of several cytokines with important consequences in the HIV pathogenesis. In addition, Tat is able to enter cells and localize to the nucleus to regulate the expression of several cellular promoters through TAR-independent mechanisms [Taylor et al., 1992].

Tat has pleiotropic biological effects on the survival, proliferation, and function of different cell lineages such as T lymphocytes, macrophages, neurons, and endothelial cells [Ensoli et al., 1993; Park et al., 2001; Barillari and Ensoli, 2002]. These Tat-related effects strongly reinforce clinical and epidemiologic studies that have correlated HIV replication with the progressive impairment of the central nervous system (CNS), bone, kidney, and the cardiovascular system in addition to immune system damage, the hallmark of HIV infection [Levy, 2009]. HIV infection is an independent risk factor for atherosclerosis and HIV-1 naïve patients exhibit a significantly increased incidence of early coronary, peripheral and cerebral atherosclerotic lesions with cardiovascular diseases and pulmonary hypertension [Mu et al., 2007; Lorenz et al., 2008; Hsue et al., 2009] compared to HIV-negative individuals. Interestingly, the formation of atherosclerotic lesions can be accelerated by treatment with protease inhibitors (PIs) suggesting a complex scenario of atherosclerosis development in HIV-positive subjects [Carr et al., 1999]. Although the relationship between HIV infection and atherosclerosis enhancement is well understood [Hakeem et al., 2010], the mechanisms involved have not been clearly elucidated, although chronic inflammation and endothelial layer impairment have been related to atherosclerosis induction during the course of HIV infection. Indeed, HIV elicits chronic immune activation and systemic inflammation along with a disruption of the regulation of different cytokines [Barqasho et al., 2009] including IL-1, IL-6, M-CSF, IL-10, TNF-α, and RANKL that may affect the atherosclerosis genesis. In addition, HIV can productively infect endothelial cells although their permissivity to HIV infection is related to endothelial anatomical origin [Kanmogne et al., 2001]. HIV gp120 elicits apoptosis of endothelial cells by caspase activation whereas Tat induces apoptosis or the proliferation of endothelial cells depending on the Tat concentration, the presence of specific pro-inflammatory cytokines and endothelial anatomical origin [Ullrich et al., 2000; Park et al., 2001]. Interestingly, Tat is also involved in the development of Kaposi's sarcoma, although its enhancing activity is detected only in the presence of basic FGF (bFGF) and specific inflammatory cytokines [Barillari et al., 1999ab].

Some reports have demonstrated that endothelial layer homeostasis is also modulated by pluripotent vessel wall mesenchymal stem cells (MSCs) that can differentiate into endothelial cells under specific stimuli [Ergun et al., 2008; Pasquinelli et al., 2010]. HIV and gp120 act on vessel wall MSCs by impairing certain differentiation pathways [Gibellini et al., 2011] and some studies performed on bone marrow-derived MSCs have demonstrated that several viral proteins, such as Tat, gp120, Rev, and p55, altered MSC differentiation [Cotter et al., 2008, 2011]. We studied the effects of Tat treatment on MSC survival and differentiation to adipocytes and endothelial cells to analyze whether Tat may be involved in the pathogenesis of the vessel structure damage observed in HIV-infected patients.

## MATERIALS AND METHODS

### CELL CULTURES AND CELL DIFFERENTIATION

Vessel wall-derived MSCs were isolated from human femoral arterial segments achieved from three male heart-beating donors (mean age 39 years) as previously described [Pasquinelli et al., 2007, 2010]. These vascular segments did not have the length and calibre required for clinical use. The characterization of arterial cells as MSCs was performed analyzing the protein and mRNA expression of specific MSC markers [Pasquinelli et al., 2010]. Vascular wall-derived MSCs were cultured in D-MEM (Lonza, Basel, Switzerland) plus 10% fetal calf serum (FCS; Gibco, Paisley, UK) and split every 3–4 days at approximately 70% density. The MSCs were seeded at a density of $5 \times 10^3$ cells/cm$^2$. For culture expansion, 75 and 25 cm$^2$ flasks (Becton Dickinson, Palo Alto, CA) treated with collagen were employed [Pasquinelli et al., 2007], while for the experiments, the MSCs (between passages 4 and 8) were seeded in untreated 6-well or 24-well plates (Nunc, Rochester, NY). Adipogenic differentiation was induced in confluent cells cultured as follows: three cycles of 3 days in induction medium and 3 days in maintenance medium (hMSC Adipogenic Differentiation Medium Kit, Lonza). The cells containing neutral lipids in fat vacuoles were determined by staining with fresh red oil solution (Sigma, St. Louis, MO) as previously described [Alviano et al., 2007]. The MSCs cultured only with adipogenic maintenance medium were used as negative controls for differentiation. Angiogenic differentiation was assessed using confluent cells, cultured in D-MEM (Lonza) with 2% FCS and 50 ng/ml vascular endothelial growth factor (VEGF; Invitrogen, Carlsbad, CA) for 7 days; the medium was changed every 2 days. The MSCs cultured in medium without VEGF throughout the induction period were considered the negative differentiation controls.

### MSC CULTURE TREATMENTS

Full-length HIV-1 Tat (100 ng/ml; Diatheva, Pesaro, Italy) was added at the same time as the first adipogenic differentiation induction and every 3 days thereafter when the media was refreshed. The samples were harvested on days 3, 7, 10, 14, and 21. In some experiments, a KDR antagonist, SU5416 (Sigma), and the integrin antagonist RGD Fc (Anaspec, San Jose, CA) were used at 100 nM and 25 μM concentrations, respectively, with or without Tat. Tat (100 ng/ml) and VEGF were added every 2 days during angiogenetic differentiation when medium refreshment was carried out. The cells were analyzed on day 7. As a control, differentiated MSCs were treated with HIV-1 p24 (100 ng/ml; NIBSC, London, UK) and Tat was pre-treated with rabbit anti-Tat polyclonal antibody (pAb, 5 μg/ml, NIBSC) or with rabbit anti-p24 pAb (5 μg/ml; NIBSC). In all experiments, unless otherwise specified, Tat was added at 100 ng/ml a concentration used in previous experiments with bone marrow-derived MSCs [Cotter et al., 2008, 2011]. In the experiments with Tat peptides (amino acid residues 1–20, 11–30, 21–40, 31–50, 41–60, 51–70, 61–80, and 74–86; all supplied by NIBSC) 100 ng/ml of each peptide was added employing the same procedure used for full-length Tat.

### PPARγ ACTIVITY ASSAY

PPARγ transcription factor activity was detected using a TransAM PPARγ kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol. This highly sensitive ELISA assay provides, after the extraction of nuclear proteins, the determination of PPARγ binding on specific consensus sequence fixed on plate wells. This binding was detected by a specific anti-PPARγ mAb revealed by an HRP-conjugated secondary pAb. The assay was read by a spectrophotometer at 450 nm. Data were compared with a reference curve following protein concentration normalization.

### FLOW CYTOMETRY ANALYSIS OF ENDOTHELIAL MARKERS

To assay the expression of endothelial specific markers (Flt-1, KDR, and vWF) by flow cytometry, $2 \times 10^5$ MSCs were analyzed at day 7 after detachment with trypsin. The samples were treated with FITC-Flt-1 mAb (1/20 dilution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) or FITC-KDR mAb (1/20 dilution in PBS; R&D System, Minneapolis, MI) for 20 min at room temperature. To detect vWF, the MSCs were permeabilized with an Intraprep Kit (Beckman-Coulter, Fullerton, CA), incubated with anti-vWF mAb (1/20 dilution in PBS; DakoCytomation, Glostrup, Denmark) for 1 h at room temperature and then incubated with FITC-conjugated anti-mouse IgG (1/40 dilution in PBS; DakoCytomation) for 30 min at room temperature. The fluorescence intensity data for intracellular and surface proteins were acquired using a Cytomics FC500 Flow Cytometer (Beckman-Coulter) and analyzed using the CXP Software (Beckman-Coulter).

### APOPTOSIS DETERMINATION

Scalar concentrations of Tat (0–1,000 ng/ml) were added to subconfluent undifferentiated MSCs or confluent MSCs differentiated toward adipogenesis or endotheliogenesis. Apoptosis analysis was performed using flow cytometry on the MSCs on days 1, 3, and 7 post-Tat treatment as previously described [Gibellini et al., 2005b]. In brief, trypsin-detached MSCs were fixed in cold 70% ethanol for 15 min at 4°C and after being washed in PBS, the samples were treated with RNase (500 μg/ml, Sigma) and then stained with propidium iodide (50 μg/ml, Sigma). The samples were analyzed with a flow cytometer (FACScan, Becton-Dickinson) equipped with an argon laser (488 nm) and Lysis II software (Becton-Dickinson).

### QUANTITATIVE REAL-TIME RT-PCR (qRT-PCR)

The mRNA expression of specific cellular genes involved in the endothelial and adipogenic differentiation was determined using the SYBR-Green qRT-PCR technique. Total RNA (100 ng) was retro-transcribed and amplified using Quantitect SYBR Green RT-PCR kit (Qiagen) and 400 nM of each specific oligonucleotide. The amplification was performed with a retro-transcription step at 50°C for 20 min, followed by initial activation of HotStar Taq DNA Polymerase at 95°C for 15 min and 40 cycles in three steps for C/EBP β, C/EBP δ, PPARγ, vWF, and KDR: 94°C for 10 s, 60°C for 15 s, and 72°C for 30 s. For Flt-1, an additional step was added at 78°C for 2 s to analyze the fluorescence. The relative quantifications were performed using specific standard external curves and the data were normalized by parallel amplification of ribosomal 18S as described previously [Gibellini et al., 2010]. All amplifications employed oligonucleotide pair sequences reported previously [Gibellini et al., 2011].

### STATISTICAL ANALYSIS

The data are expressed as the mean ± standard deviation (±SD) of three separate experiments performed in duplicate. A statistical analysis was performed using a two-tailed Student's *t*-test.

## RESULTS

### HIGH CONCENTRATIONS OF TAT INDUCE APOPTOSIS IN SUBCONFLUENT VESSEL WALL-DERIVED MSCs

In the first set of experiments, we assayed whether extracellular Tat affects MSC survival. Scalar concentrations of Tat (0–1,000 ng/ml) were added to subconfluent MSC cultures and the apoptosis activation was analyzed with a flow cytometry procedure on days 1, 3, and 7 post-treatment. Significant induction of apoptosis was detectable ($P < 0.05$) at days 3 and 7 only at higher concentrations of full-length recombinant Tat (200–1,000 ng/ml), whereas 1–10 ng/ml Tat induced a slight but not significant decrease of apoptosis (Fig. 1A). Treating MSCs with p24 or Tat pre-treated with anti-Tat pAb did not have any significant effect on cell survival (Fig. 1B). When the same experimental protocol was performed with confluent MSCs differentiated either to adipocytes or endothelial cells, apoptosis induction was not detectable, even at higher Tat concentrations (data not shown).

### TAT ENHANCES THE MSC DIFFERENTIATION TO ADIPOCYTES

Vessel wall-derived MSCs can differentiate to several cell lineages including adypocytes, osteoblasts, chondroblasts, smooth muscle, and endothelial cells [Pasquinelli et al., 2010]. A recent analysis of bone marrow or vessel wall-derived MSCs revealed an increase of adipogenesis when these cells were challenged by HIV or treated with gp120 during the differentiation induction [Cotter et al., 2011; Gibellini et al., 2011]. In addition, in bone marrow MSCs, Tat elicited a further activation of adipogenesis [Cotter et al., 2011]. As the anatomical origin of MSCs can be correlated with different biological responses to normal or pathological stimuli [Montesinos et al., 2009], we investigated whether Tat modulated adipogenesis development in vessel wall MSC differentiation.

Tat (100 ng/ml) was added during the first adipogenic induction treatment and every 3 days post-induction during medium replacement. Adipogenic differentiation was tested via red oil staining of cell cultures. The microscopic analysis of cell cultures, performed at days 7 and 10, indicated a Tat-induced enhancement of adipogenesis. Tat-treated differentiated cell cultures exhibited an increase of red oil positive cells compared to Tat-untreated differentiated MSCs (Fig. 2). As expected, p24 or Tat pre-treated with anti-Tat pAb did not result in any increase in adipogenesis (Fig. 2). Interestingly, Tat treatment of undifferentiated MSCs did not induce any adipogenesis differentiation in MSC cell cultures, indicating that Tat enhanced adipogenesis only in the presence of differentiation medium (data not shown).

To confirm the red oil staining data, we analyzed the expression of specific factors involved in the early stages of adipogenesis such as PPARγ, C/EBPβ, and C/EBPδ. PPARγ is a master regulator of

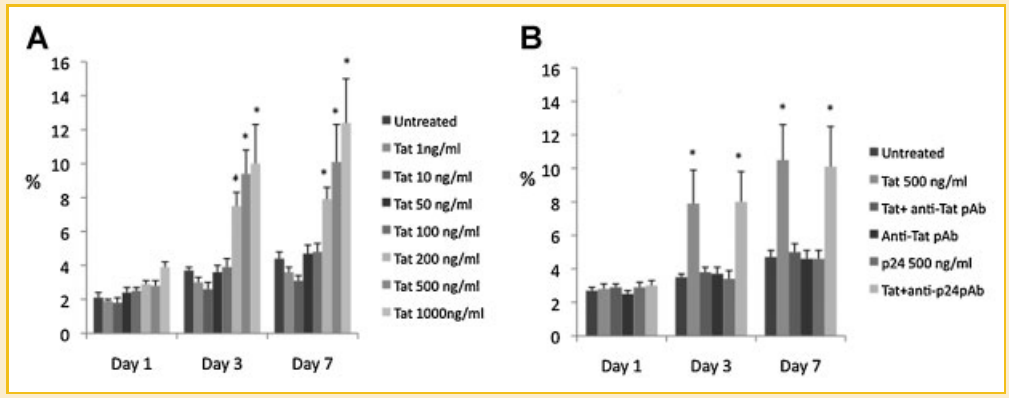


Fig. 1. High concentrations of Tat elicit MSC apoptosis. In (A), scalar concentrations of Tat (0–1,000 ng/ml) were added to subconfluent MSC cultures and the number of apoptotic cells was determined by flow cytometry at days 1, 3, and 7. A significant increase in apoptosis induction is detectable in MSCs at days 3 and 7 only when higher Tat concentrations are used (200–1,000 ng/ml of Tat). In (B), apoptosis analysis of MSCs treated with Tat (500 ng/ml) that was pre-treated with rabbit polyclonal anti-Tat or p24 (500 ng/ml) was performed. Three experiments performed in triplicate were carried out. Statistical significance was determined using the Student's *t*-test with $^{*}P < 0.05$.

adipogenesis and its activity was assayed at day 6 using an ELISA TransAm assay (Fig. 3A). Tat elicited a significant increase of PPARγ activity in comparison with the Tat-untreated control ($3.9 \pm 0.3$-fold increase, $P < 0.05$). This effect was abolished when Tat was pre-treated with anti-Tat pAb. Although PPARγ is mainly regulated post-transcriptionally by phosphorylation, a quantitative analysis of PPARγ mRNA content by qRT-PCR was carried out. Tat upregulated PPARγ mRNA expression ($2.9 \pm 0.5$-fold increase, $P < 0.05$) compared to differentiated cell culture controls (Fig. 3B).

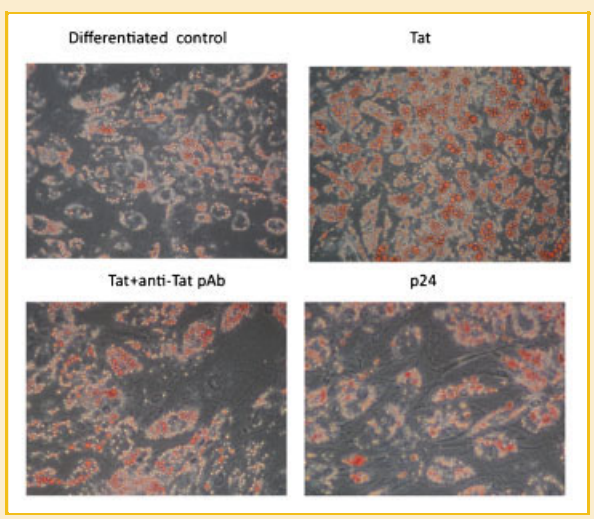


Fig. 2. Red oil staining of MSCs differentiated towards adipogenesis at day 10. MSCs challenged with Tat (100 ng/ml) displayed more abundant multi-vacuole adipogenic vesicles in the cytoplasm than differentiated control cells. The addition of neutralizing anti-Tat pAb plus Tat (100 ng/ml) or p24 (100 ng/ml) treatment in MSC samples did not result in any significant increase in the number of red oil stained lipid drops. A typical experiment is shown. Magnification 200×. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

In parallel, we also investigated the expression levels of C/EBPβ and C/EBPδ mRNA because these genes play an important role in the earliest differentiation commitment to adipogenesis. The qRT-PCR data (Fig. 3E) indicated that Tat upregulates the mRNA expression of these two genes ($10.3 \pm 2.5$, $P < 0.05$ for C/EBPβ and $3.9 \pm 1.1$, $P < 0.05$ for C/EBPδ at day 3).

As previously reported [Cotter et al., 2011], MSCs have some major Tat receptors such as the KDR and integrin $\alpha_v\beta_3$ receptors. To investigate whether Tat binding with the KDR and integrin $\alpha_v\beta_3$ receptors is involved in the Tat-mediated enhancement of adipogenesis, we treated cell cultures with SU5416 or RGD-Fc, which are antagonists of KDR and integrin receptors, respectively [Cotter et al., 2011]. Interestingly, both SU5416 and RGD-Fc treatment determined a strong attenuation of Tat-related PPARγ activity and expression induction (Fig. 3C,D).

## TAT INHIBITS MSC DIFFERENTIATION TO ENDOTHELIAL CELLS

Vessel wall derived-MSCs can be differentiated to endothelial cells by VEGF treatment. Hence, in the next series of experiments, we have evaluated whether Tat can affect this differentiation. MSCs were treated by VEGF with or without Tat and the mRNA expression levels of specific endothelial differentiation markers, represented by vWF, Flt-1, and KDR, were determined at day 7. The qRT-PCR analysis indicated that Tat induced a significant inhibition in the mRNA content of all three endothelial differentiation markers ($P < 0.05$; Fig. 4). These results were additionally confirmed by flow cytometry analysis of the protein expression of these vWF, Flt-1, and KDR (Fig. 5 and data not shown). In particular, Tat induced a significant downregulation of intracellular vWF protein (Fig. 5) whereas MSCs treated with p24 or Tat pre-treated with neutralizing anti-Tat pAb did not produce any significant biological effect on mRNA and protein endothelial marker expression. It is noteworthy that the expression of vWF is inversely related to Tat protein concentration (Fig. 6). In fact, vWF inhibition is clearly detectable when 10–100 ng/ml of Tat was used. These data indicate that this Tat-related negative differentiation effect might be consistent when HIV replication levels are high.

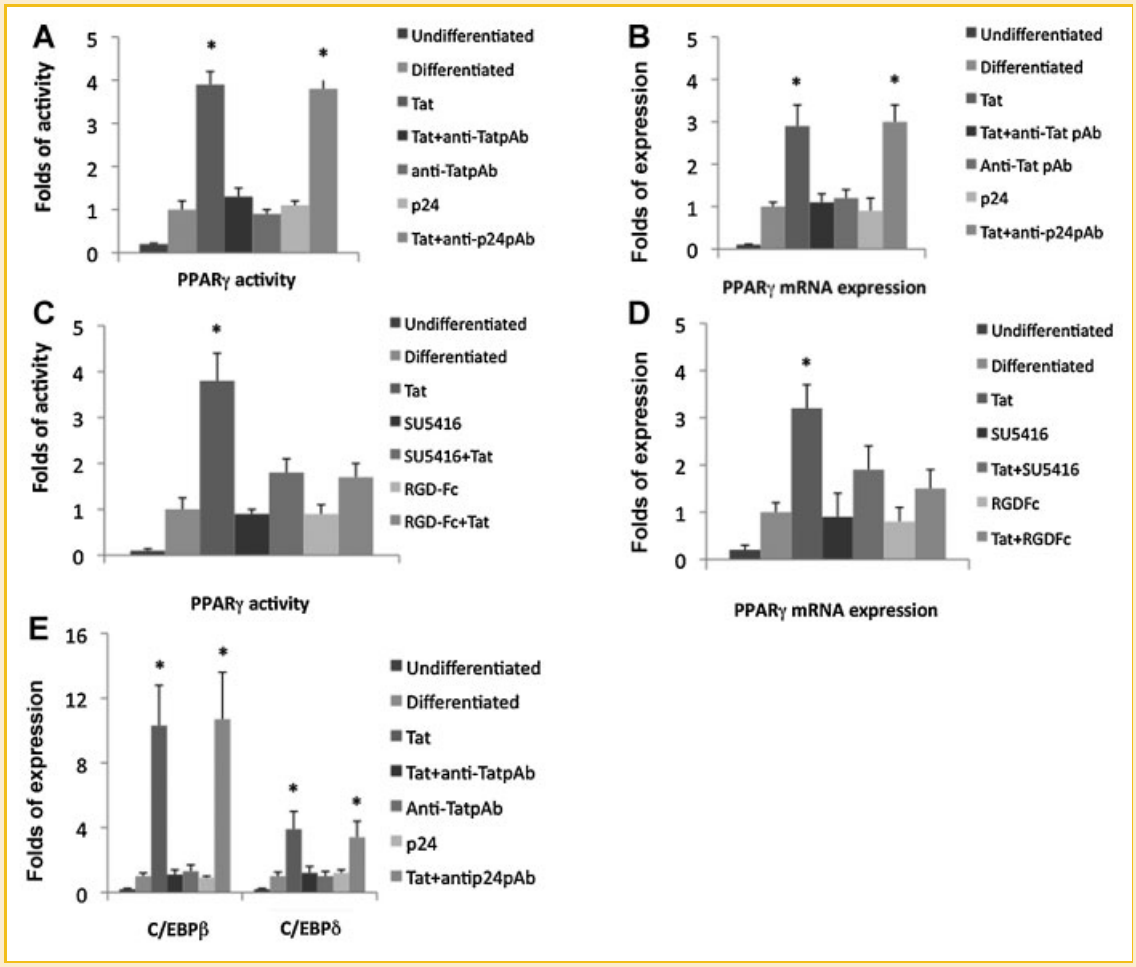


Fig. 3. Effect of Tat on activity and mRNA expression of adipogenesis-related transcription factors in MSCs differentiated to adipocytes. In (A) and in (C), MSCs were challenged with Tat (100 ng/ml) in the presence or absence of anti-Tat pAb, SU5416, or RGD-Fc. MSCs were harvested at day 6, and nuclear extracts were analyzed for PPARγ activity using a TransAM PPARγ kit. The PPARγ activity data are expressed as the ratio (±SD) between samples and the control represented by MSC cell cultures differentiated to adipocytes. The adipogenesis-differentiated cell culture PPARγ activity was set to 1. Three experiments performed in duplicate were carried out. In (B,D), quantitative real-time RT-PCR was performed at day 6 to analyze PPARγ mRNA expression in cell cultures treated with Tat. In (E), MSCs were treated with Tat (100 ng/ml) in the presence or absence of anti-Tat pAb and anti-p24 pAb. The figure depicts the upregulation of C/EBPβ and C/EBPδ, which are involved in the early adipogenetic differentiation. The analysis was performed at day 3 post-stimulation. The C/EBPβ and C/EBPδ mRNAs were significantly increased in Tat-treated adipogenesis-differentiated samples with respect to Tat untreated adipogenesis-differentiated control cell cultures. The mRNA expression data are shown as the ratio between samples and the control represented by adipogenesis-differentiated cell cultures after 18S ribosomal normalization. The adipogenesis-differentiated cell culture mRNA level of specific genes was set to 1. Three experiments performed in duplicate were carried out. Statistical significance was determined using Student's *t*-test with $^{*}P < 0.05$.

To investigate the Tat-related mechanisms of endothelial differentiation downregulation, we analyzed the vWF protein expression in VEGF-differentiated MSCs treated with Tat fragments. Eight overlapping peptides corresponding to 20 amino acids of the HIV-1 Tat sequence were added to MSCs differentiated to endothelial cells by VEGF. The peptides representing the Tat residues 61–80 and 74–86, which encompass the RGD sequence in the second exon, downregulated vWF expression. Interestingly, even peptides expressing the basic domain (Tat 41–60 and Tat 51–70) or the complete cysteine-rich region (Tat 21–40) demonstrated a reliable decrease of vWF protein although to a lesser extent than Tat residues expressing RGD domain. The remaining Tat peptides (Tat 1–20 and Tat 11–30) induced no effects on vWF protein expression (Fig. 7), indicating that specific Tat domains were involved in the negative modulation of MSC differentiation to endothelial cells.

## DISCUSSION

In this report, we analyzed the biological effects of Tat treatment on vessel wall-derived primary MSCs and in their differentiation into adipocytes and endothelial cells. The results of our study demonstrated that: (i) high concentrations of Tat induced apoptosis in subconfluent primary vessel wall-derived MSCs; (ii) Tat enhanced the vessel wall-derived MSC differentiation to adipocytes via an increase in PPARγ expression and activity; (iii) Tat negatively affected the VEGF-driven vessel wall-derived MSC differentiation to endothelial cells and this differentiation impairment is due to specific Tat domains.

In the first set of experiments, we found that Tat treatment of subconfluent primary vessel wall-derived MSCs induced apoptosis when high Tat concentrations were employed (200–1,000 ng/ml),

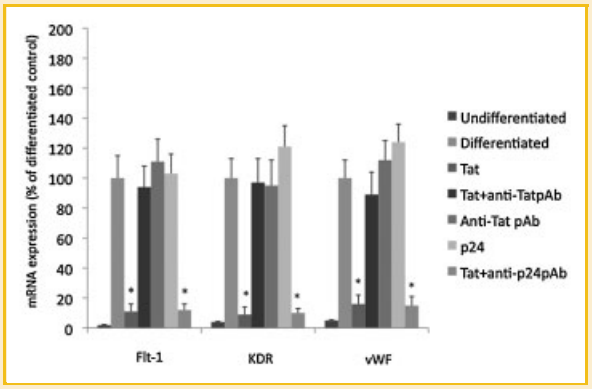


Fig. 4. Determination of vWF, Flt-1, and KDR mRNA by qRT-PCR. MSCs were differentiated to endothelial cells by VEGF and treated with Tat (100 ng/ml), Tat plus anti-Tat pAb (5 μg/ml), Tat plus anti-p24 pAb (5 μg/ml), or p24 (100 ng/ml). Total RNA was extracted and purified at day 7 and vWF, Flt-1, and KDR mRNAs were analyzed. The results are expressed as the ratio between samples and the control represented by VEGF-differentiated cell cultures after 18S ribosomal normalization. The VEGF-treated control cell culture mRNA level was set to 100. The analysis was performed at day 7. The data represent the mean (±SD) of three independent experiments performed in duplicate. Statistical significance was determined using Student's *t*-test with $^{*}P < 0.05$.

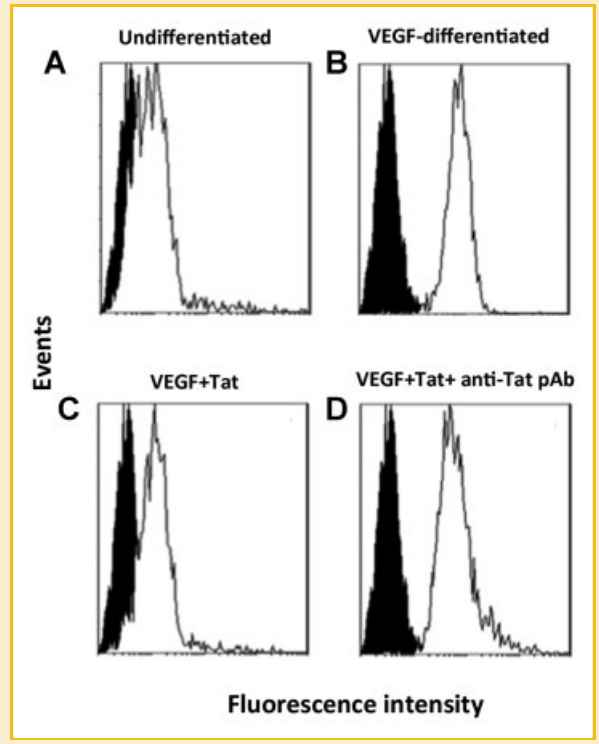


Fig. 5. Tat inhibits vWF protein expression in MSCs. Panels A and B represent the flow cytometry analysis of vWF intracellular protein expression in undifferentiated and VEGF differentiated MSCs. Panels C and D show the vWF protein expression of VEGF-differentiated MSCs treated with Tat (100 ng/ml), or Tat (100 ng/ml) plus rabbit anti-Tat pAb (5 μg/ml), respectively. The VEGF-induced expression of vWF intracellular protein was strongly inhibited by Tat. The analysis was performed at day 7. The shadowed areas represent samples treated with irrelevant mAb plus FITC-conjugated secondary antibody, whereas the non-shadowed areas are the MSCs stained with anti-vWF mAb plus FITC-conjugated secondary antibody. A typical experiment is shown.

whereas lower concentrations of Tat (1–10 ng/ml) provided a slight but not significant decrease of programmed cell death. Apoptosis induction is a major pathway in HIV pathogenesis [Levy, 2009]. In addition to HIV infection and gp120 [Zauli et al., 1996; Lawson et al., 2004], even Tat is involved in the modulation of cell proliferation and survival by induction of cellular pro-apoptotic or anti-apoptotic responses that vary depending on the cell type and Tat concentration [Zauli and Gibellini, 1996; Milani et al., 1998; Barillari and Ensoli, 2002]. Tat can promote cell survival and proliferation in some cell lineages at picomolar/nanomolar concentrations, whereas, at higher concentrations (nanomolar/micromolar), Tat induces apoptosis. Recent observations have indicated that MSC apoptosis is also induced by HIV-1 and gp120 through a direct interaction with the cell membrane [Gibellini et al., 2011]. Tat may also support an additional apoptosis route although this cooperation could be available in situations where high degree of HIV replication occurred as in naïve HIV patients. Interestingly, Tat (even at the highest tested concentrations) did not induce apoptosis activation during the differentiation to adipocytes or endothelial cells. This phenomenon might be explained by differentiation stimuli activity that strongly promotes cell survival by inhibiting Tat apoptosis activation. For example, VEGF induces cell survival, which is necessary for subsequent differentiation through the positive regulation of AKT/PI-3-kinase signal transduction pathway [Grünewald et al., 2010].

Vessel wall-derived MSCs can differentiate towards several cell lineages [Pasquinelli et al., 2007]. Thus, we studied the Tat-related effects on MSC differentiation to adipocytes and endothelial cells. Our data displayed that Tat enhanced the adipogenesis induction as documented by red oil staining of cell cultures. In addition, Tat increased the expression of C/EBPβ and C/EBPδ, two transcription factors that play a pivotal role in the early steps of adipogenesis. The linkage between Tat and the C/EBP family factors has already been described in other cellular targets than MSCs [Liu et al., 2002; Mameli et al., 2007]. Tat induced C/EBPβ expression and cooperated in LTR promoter modulation with C/EBP family members to increase the HIV transcription [Liu et al., 2002]. C/EBPβ and C/EBPδ factors also modulated the expression of PPARγ [Yeh et al., 1995] whose transcription and, more evidently, activity were enhanced by Tat in MSCs during adipocyte differentiation. PPARγ is a key factor in the adipogenesis because it regulates the expression of several genes activated during adipocyte differentiation through the PPAR response elements [Lehmann et al., 1995]. Our results are in accordance with a previous report indicating that Tat increases the adipogenesis in bone marrow-derived MSCs by downregulation of COUP-TF1 factor, a negative regulator of PPARγ [Cotter et al., 2011]. Interestingly, the upregulation of PPARγ activity and adipogenesis enhancement were also shown in bone marrow MSCs by treatment with other HIV-related molecules, such as gp120 and p55 [Cotter et al., 2008, 2011; Gibellini et al., 2011], indicating that PPARγ can be considered the pivotal target of viral induction of adipogenesis commitment. The Tat-related enhancement of adipogenesis is tackled by treatment with antagonists of KDR and integrin receptors. These data support the article by Cotter et al. [2011] in

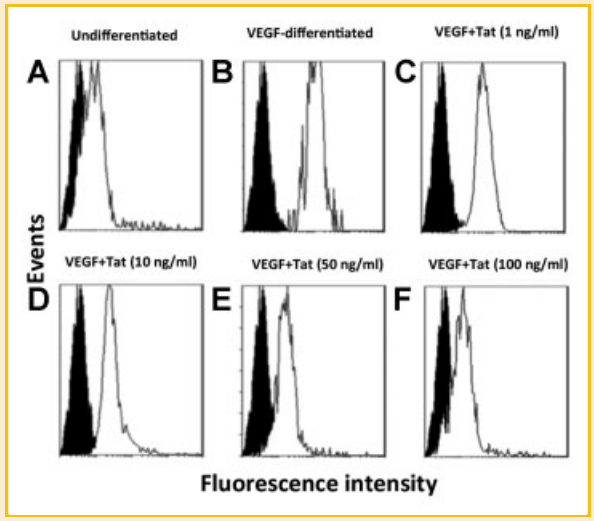


Fig. 6. Flow cytometry analysis of vWF intracellular protein in MSC treated by scalar concentrations of Tat (0–100 ng/ml). Panel A displays undifferentiated MSC cells. Panels B–F represent VEGF-differentiated MSCs treated with 0, 1, 10, 50, and 100 ng/ml of Tat, respectively. Tat negatively affected the modulation of intracellular vWF protein in VEGF-differentiated cell cultures already at 10–50 ng/ml. The analysis was performed at day 7. The shadowed areas represent samples treated with irrelevant mAb plus FITC-conjugated secondary antibody, whereas the non-shadowed areas are the MSCs stained with anti-vWF mAb plus FITC-conjugated secondary antibody. A typical experiment is shown.

bone marrow-derived MSCs, where Tat-related induction of adipogenesis was inhibited by these antagonists, and suggest a consistent and reliable Tat adipogenesis activity that is independent of MSC anatomical origin.

We also evaluated the impact of Tat on MSC differentiation to endothelial cells by demonstrating that Tat inhibited the expression of endothelial markers in VEGF-driven MSC differentiation. Confluent MSCs were sensitive to VEGF differentiation mediated by KDR activation, as Flt-1 was not detectable by flow cytometry on the MSC membrane even though mRNA expression was detected by RT-PCR [Pasquinelli et al., 2010]. This interaction between VEGF/KDR leads to activation of endothelial differentiation, with a subsequent increase of KDR expression and induction of vWF and cell membrane Flt-1, which positively modulate the endothelial differentiation [Pasquinelli et al., 2010].

It is noteworthy that the relationship between Tat and VEGF was analyzed in several cell systems, including endothelial and Kaposi's sarcoma-derived spindle cells [Barillari and Ensoli, 2002]. Interestingly, in these cellular models, Tat synergized with bFGF and some inflammatory cytokines to induce proliferation, vascular permeability, and cellular migration [Barillari et al., 1993, 1999ab; Albini et al., 1996; Urbinati et al., 2005] but Tat did not have these biological effects when added alone [Barillari et al., 1999a]. This Tat activity is mediated by the binding of the basic domain and RGD sequence with KDR and $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, respectively, although additional activation pathways cannot be excluded [Barillari and Ensoli, 2002]. Strikingly, Tat did not synergize with VEGF in promoting endothelial cell proliferation and VEGF blocks Tat-induced endothelial migration [Albini et al., 1996; Jia et al., 2001; Barillari and Ensoli, 2002] but in vivo Tat induces VEGF expression in mouse tissue with the concomitant addition of inflammatory cytokines [Barillari et al., 1999b]. In VEGF-treated MSCs, the treatment with Tat peptides indicated that fragments encompassing full basic, cysteine-rich, and RGD domains down-regulated to different extents the expression of endothelial markers.

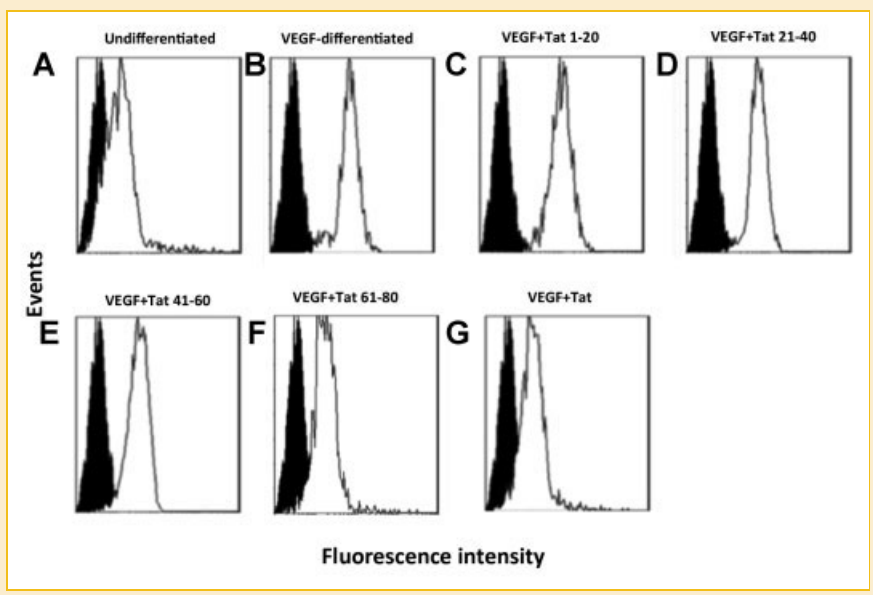


Fig. 7. Analysis of vWF intracellular protein in MSC treated by Tat and Tat fragments. MSCs were treated with full-length Tat (100 ng/ml) or with specific Tat peptides (100 ng/ml) encompassing the whole protein sequence of Tat. The treatment with Tat peptides expressing the RGD sequence (Tat residues 61–80 and also 74–86) placed in the second Tat exon promoted the inhibition of VEGF-induced vWF expression. Tat residues 21–40 and 41–60 peptides, containing the cysteine-rich and basic domain, respectively, induced vWF inhibition even though to a lesser extent, whereas no effect was observed following treatment with N-terminal Tat peptides (Tat residues 1–20 and 11–30). A typical experiment is shown.

Basic and cystein-rich domains represent the Tat region involved in the recognition of KDR whereas the RGD Tat sequence binds $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. The mechanism(s) involved in this differentiation inhibition may be related either to a possible binding competition between Tat and VEGF [Jia et al., 2001], or a heterodimerization of VEGF/Tat that reduces the activity of VEGF [Barillari and Ensoli, 2002]. In addition, Tat has a heparan-binding property that could affect the interaction between heparan sulfate proteoglycans and VEGF, thus inhibiting optimal VEGF/KDR binding [Rusnati et al., 1997]. Additional reports have demonstrated that Tat competes for vitronectin binding with $\alpha_v\beta_3$ integrins, which play an important role in full activation of VEGF [Barillari et al., 1993]. All these putative mechanisms may be involved to different extents in the inhibition of MSC differentiation to endothelial cells; however, the possibility of signal transduction pathway activations, due to Tat binding to these receptors, that inhibited the differentiation, cannot be ruled out. Further studies are needed to clarify the regulation of Tat/VEGF biological activity in MSCs.

This Tat-induced derangement of MSCs in vessel walls may be considered as an additional mechanism involved in the formation of vascular lesions and fat tissue that is observed during HIV infection. HIV strongly affects fat cells and the lipidic metabolism that determines lipid imbalance and lipodystrophy [Brown, 2011]. Low HDL cholesterol and high triglycerides levels have been shown in over 50% of naïve subjects [Grunfeld et al., 1989]. The triglyceride increase is mediated by inflammatory cytokines (IFNα, TNFα, and IL-1) that increase hepatic fatty acid synthesis and VLDL production [Grunfeld et al., 1992] whereas the mechanism(s) of HDL decline is not clear but it has been speculated that the low levels of HDL particles may result in an increase in oxidized LDL concentrations, pre-disposing the patient to atherosclerosis [Hsue et al., 2009]. HIV-related atherosclerosis is also related to the direct role of inflammatory cytokines on monocytes and cholesterol metabolism. Some reports indicate that monocytes are the precursors of the lipid foam cells detectable within the atherosclerotic plaque. These foam cells produce IL-6, a cytokine positively regulated by Tat in monocytes [Barqasho et al., 2009; Crowe et al., 2010], promoting plaque expansion with vascular cell degeneration and apoptosis [Crowe et al., 2010]. These lesions are exacerbated by HIV infection and endothelial cell apoptosis causing an impairment of vascular structure and the endothelial layer [Park et al., 2001; Mu et al., 2007; Crowe et al., 2010]. Cardiovascular disease and accelerated atherosclerosis development have been detected in the peripheral, coronary and cerebral arteries independently of traditional atherosclerosis risk factors [Lorenz et al., 2008]. The incidence of cardiovascular diseases increases two- to threefold in HIV seropositive patients compared to selected control group patients [Hsue et al., 2009; Klein et al., 2010].

This alteration of MSC differentiation may result in the progressive loss of vascular homeostasis, thus promoting the atherosclerosis genesis and the development of cardiovascular damage. Interestingly, Tat might synergize with gp120. The envelope protein also elicits similar effects on the differentiation of bone marrow and vessel wall-derived MSCs [Cotter et al., 2008, 2011; Gibellini et al., 2011]. As gp120 and Tat are present in a free form in the blood and tissues, high levels of HIV replication such as what is observed in naïve patients, might lead to the disruption of vessel wall homeostasis and a polarization of MSC differentiation due to vessel wall derived-MSCs being biased towards adipogenesis as opposed to endothelial differentiation.

These findings might indicate that vessel homeostasis by MSCs could be compromised with a progressive loss of repair effectiveness in the endothelial layer and an imbalanced differentiation in the vessel structure. In addition, MSC differentiation dysregulation might also explain the fat tissue observed in atherosclerotic vessel degeneration [Tang et al., 2005] and it should be intriguing to analyze in further studies whether MSCs may be involved in foam cell genesis and/or regulation. In conclusion, the Tat-induced derangement of MSCs and their differentiation could be considered an additional important mechanism involved in promoting vascular damage and atherosclerosis in the course of HIV disease.

## ACKNOWLEDGMENTS

We thank NIBSC for p24, anti-Tat pAb, and anti-p24 pAb reagents. This study was funded by Fondazione Cassa di Risparmio Bologna, Italy (n° 2006.0035, June 2006). Fondazione del Monte di Bologna e Ravenna, Italy (n° 376 bis/2009). AIDS projects of the Italian Ministry of Health (n° 40H5). Funds for selected research topics of the University of Bologna and MURST 60% (n°RFO09GIBEL).

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